

Therapeutic Effect of Blueberry Extracts for Acute Myeloid Leukemia

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Abstract

Acute Myeloid Leukemia (AML) is an aggressive hematological malignancy with high incidence in the aging population. In addition, AML is one of the more common pediatric malignancies. Unfortunately, both of these patient groups are quite sensitive to chemotherapy toxicities. Investigation of blueberries specifically as an anti-AML agent has been limited, despite being a prominent natural product with no reported toxicity. In this study, blueberry extracts are reported for the first time to exert a dietary therapeutic effect in animal models of AML. Furthermore, in vitro studies revealed that blueberry extracts exerted anti-AML efficacy against myeloid leukemia cell lines as well as against primary AML, and specifically provoked Erk and Akt regulation within the leukemia stem cell subpopulation. This study provides evidence that blueberries may be unique sources for anti-AML biopharmaceutical compound discovery, further warranting fractionation of this natural product. More so, blueberries themselves may provide an intriguing dietary option to enhance the anti-AML efficacy of traditional therapy for subsets of patients that otherwise may not tolerate rigorous combinations of therapeutics.

Keywords

Acute myeloid leukemia; Blueberry; Mouse models

Introduction

Natural product research has described numerous plant-derived compounds and extracts with beneficial health effects. Blueberries are rich sources for polyphenols, and are generally recognized for their health benefits arising from studies in models of aging, inflammation, diabetes, and cancer [1-5]. In contrast, the efficacy of blueberries or their components in cellular models of Acute Myeloid Leukemia (AML) and other leukemia's has minimally been explored [6]. AML is a heterogeneous group of hematological cancers defined by clonal expansion of immature myeloid progenitors in the blood and bone marrow [7-9]. It is an aggressive disease that is defined by various potential cytogenetic and molecular alterations and immunophenotypes [10]. While the incidence of AML is greatest in the aging population, it can occur at any age and is one of the more common pediatric malignancies [7-9]. The prognosis for most AML is poor, with minimal clinical advances being made over the past few decades [10]. Remission is sometimes achieved, but only after aggressive chemotherapy that can be detrimental to pediatric and elderly patients. Therefore, studies aimed at identifying bioactive compounds with anti-AML efficacy from blueberries and other natural products are of interest for biopharmaceutical development given the need for effective and minimally toxic therapies. In the current study, we evaluated an anti-AML effect for blueberry extracts in both cellular and animal models of AML. By defining a role for blueberries as an anti-AML natural product this study may also lead to dietary interventions to augment the treatment of AML in pediatric or elderly patients that otherwise may not respond well to aggressive chemotherapy.

Materials and Methods

Cell culture

Murine C1498, 32D-p210-GFP, and 32D-FLT3-ITD cells, and human HL-60/vcr cells were maintained at 37°C, and 5% CO₂, in RPMI-1640 supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. Human KG-1 cells were likewise maintained, in IMDM supplemented with 20% FBS and 1% penicillin/streptomycin. Patient AML

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samples were obtained using informed consent approved by the Penn State College of Medicine Institutional Review Board. Samples were prepared from peripheral blood or bone marrow using Ficoll-Paque separation of white blood cells, and for short term assays were maintained in RPMI-1640 supplemented with 10% FBS.

In Vitro Assays

Cellular viability assays were performed as previously described using a Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay according to the manufacturer's instructions (Promega, Madison, WI) [11,12]. Colony forming assays were performed as previously described using MethoCult H4434 according to the manufacturer's instructions (Stem Cell Technologies, Vancouver, BC, Canada) [11]. Apoptosis assays were performed as previously described using Annexin V and 7-aminoactinomycin D from BD Biosciences (San Jose, CA), and a fluorophore-conjugated antibody targeting CD45 from Biolegend (San Diego, CA) to gate the blast population [11,12]. Phosflow analysis was performed by using the BD Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Biosciences, San Jose, CA). Briefly, cells were fixed following treatment and then their membranes were permeabilized to permit intracellular staining with fluorophore-conjugated antibodies targeting phosphorylated Akt and Erk (BD Biosciences, San Jose, CA). Cells were subsequently stained to evaluate leukemia stem cells with fluorophore-conjugated antibodies targeting CD34 and CD38 (BD Biosciences, San Jose, CA), as well as CD96 (eBioscience, San Diego, CA). Flow cytometry was performed at the Penn State College of Medicine Flow Cytometry Core using a BD Biosciences LSR II flow cytometer and BD FACS Diva software.

Animal trials

NOD-Scid and C57BL/6J mice were bred from founders obtained from the Jackson Laboratory (Bar Harbor, ME). Female NOD-Scid mice were xenografted with HL-60/vcr (2×10^6 cells/ mouse) or KG-1 (2.5×10^6 cells/ mouse) cells by tail vein injection. After one week, mice were randomized and given *ad libitum* access to either control drinking water or crude blueberry extract dissolved in drinking water (1% w/v). Male and female C57BL/6J mice were engrafted with C1498 cells (1×10^6 cells/ mouse) by retro-orbital injection. After one week, mice were randomized and given a crude blueberry preparation to eat (25 mg/ mouse/ week), *ad libitum* access to chloroquine in the drinking water (0.288 mg/ml sweetened with 15 mg/ml glucose to alleviate bitterness), a combination of both, or control water (sweetened with 15 mg/ml glucose) [13]. Mice were euthanized once they reached moribund status. All procedures were approved by the Institutional Animal Care and Use Committee of the Penn State College of Medicine.

Blueberry extraction

Solvents and reagents were obtained from VWR (Radnor, PA) and Sigma (St. Louis, MO). Briefly, whole blueberries were lyophilized, crushed to powder, and a crude extract was prepared by extracting for 30 minutes with agitation using aqueous acetone (70/30 acetone/ water), and dried by rotary-evaporation and lyophilization [3,4]. As these were whole extracts, the mass yield was slightly over 50% of the original starting material. Standardized blueberry powder was alternatively obtained from FutureCeuticals (Mornence, IL). No differences were noted in cell culture studies between the blueberry crude extracts, and the standardized blueberry powder.

Statistical analysis

Colony-forming and apoptosis assay comparisons of untreated controls and treatments with blueberry extracts were made using an unpaired t-test. For *in vivo* studies, survival analysis was performed using the Mantel-Cox Logrank test. All experiments were performed with an $n \geq 3$ biological replicate sample size, and graphical averages are depicted \pm standard deviation from the mean.

Results and Discussion

Initially, blueberry extracts were evaluated for anti-AML

therapeutic efficacy using cell lines and patient samples. Crude preparations were observed to diminish the viability of the AML cell lines HL-60/vcr, C1498, and 32D-FLT3-ITD, as well as the Chronic Myeloid Leukemia (CML) cell line 32D-p210-GFP (Figure 1A). Anti-AML efficacy for crude blueberry extracts was further demonstrated using a colony forming assay with a poor prognosis patient sample (#329: inv3, -7) (Figure 1B), as well as several patient samples using short-term apoptosis assays (Figure 1C). Apoptosis assays used

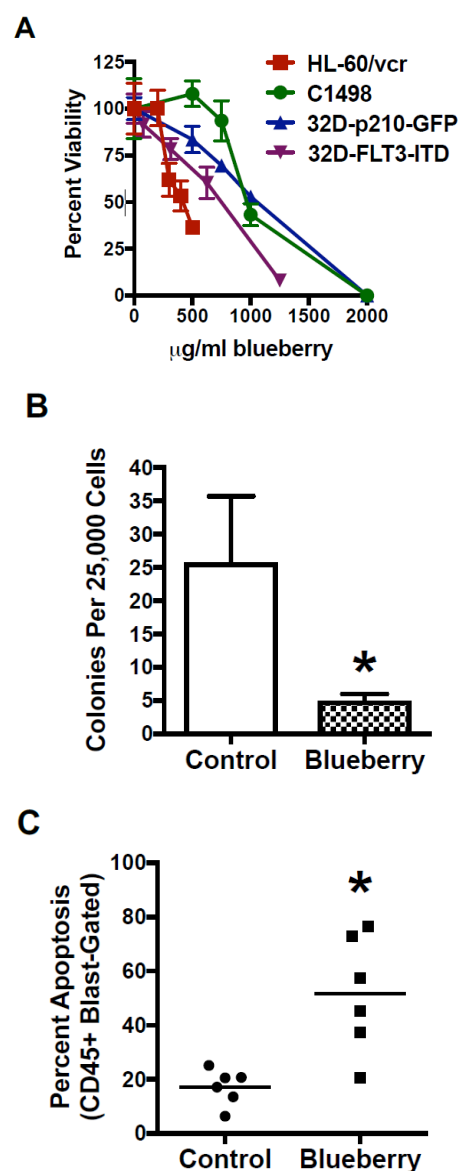


Figure 1: In vitro anti-AML therapeutic effect of blueberry extracts.

- (A) Cellular viability was determined by MTS assay following 48-hour exposure of AML cell lines (dashed line with circles: C1498, solid line with squares: HL-60/vcr, dashed line with upside down triangles: 32D-FLT3-ITD) and a CML cell line (solid line with right side up triangles: 32D-p210-GFP) to crude blueberry extract.
- (B) Colony forming capacity of a poor prognosis patient AML case #329 was determined in the presence of 500 µg/ml crude blueberry extract (t-test, $p = 0.0245$, $n \geq 3$).
- (C) Ficol-prepared WBC fractions of patient AML (case #651, #652, #657, #658, and #661) and patient CML (case #653) were exposed to 500 µg/ml crude blueberry extracts for 48 hours followed by flow cytometry to determine percent apoptosis (Annexin V+) in the CD45-gated blast population (t-test, $p = 0.0098$, $n = 6$).

several different patient cases including three poor prognosis AML (#651: -7, -5, #652: -7, #658: complex cytogenetics), one intermediate prognosis AML (#657: +8), one favorable prognosis AML (#661: inv16), and one CML case (#653). Overall, these results demonstrated *in vitro* anti-leukemia efficacy for blueberry extracts using both cell lines and primary patient samples. Further studies are warranted to identify the specific components of blueberries that exert anti-leukemia efficacy to facilitate their potential biopharmaceutical development. These constituent components of the blueberries may target signaling pathways critical to survival and proliferation of leukemia or related hematological disorders. Phosflow analysis was used to evaluate the effects of blueberry extracts on leukemia stem cells. This technique allows single cell analysis of proteins such as Akt and Erk, which are kinases critical to regulating signaling networks classically responsible for growth and survival. In this study, HL-60/vcr cells displayed dose-dependent increases in the phosphorylation of Erk, indicative of activation, in response to blueberry extract exposure (Figure 2A). This effect was evident in both the leukemia stem cells, defined as CD34+CD38- or CD34+CD38-CD96+, as well as within the bulk leukemia cell population. Previously, Hosen *et al.* used CD96 to further define the AML stem cell fraction [14]. Our group later evaluated CD96 as an AML stem cell marker for targeted therapeutics [15]. In the present research study, Phosflow analysis of a poor prognosis AML patient sample revealed that blueberry extracts augmented Erk phosphorylation, as well as Akt phosphorylation, within both the leukemia stem cell (CD34+CD38- or CD34+CD38-CD96+) and bulk leukemia cell populations (Figure 2B). This observation of an increase in Erk and Akt signaling may seem counterintuitive at first, but is specifically meaningful within the context of leukemia stem cell biology. Leukemia stem cells are quiescent and not cycling, which is important in protecting them from DNA-damaging chemotherapeutics [16,17]. Viale *et al.* demonstrated that disruption of the cell cycle inhibitor p21 caused leukemia stem cells to hyper-proliferate which ultimately depleted their population [16]. This study provided support for the concept of a two-step approach to leukemia treatment whereby initial treatment provokes the leukemia stem cell to enter the cell cycle and hyper-proliferate, followed by a second more cytotoxic therapy. In the context of the current study, where blueberry extracts represent a complex mixture of potentially therapeutic compounds, it is possible that particular compound(s) provoke the leukemia stem cell to enter the cell cycle by promoting Erk and/or Akt phosphorylation. At the same time, other blueberry compound(s) may exert a cytotoxic effect on these newly cycling leukemia stem cells as well as the overall leukemia cell population. This suggests that even blueberry extracts themselves may have utility as a therapeutic alternative or adjuvant to traditional anti-leukemia therapeutics which may be effective given the ability to provoke leukemia stem cells into an ultimately detrimental proliferative cycle. Studies to define and isolate constituent components of blueberries that influence leukemia stem cells specifically are warranted.

Anti-AML efficacy for crude blueberry extracts was next evaluated using *in vivo* models. The lifespan of NOD-Scid mice xenografted with human HL-60/vcr (Figure 3A), or KG-1 cells (Figure 3B), was significantly extended by blueberry supplemented in drinking water. Additionally, blueberry extracts formulated as an edible snack modestly extended the lifespan of C57BL/6J mice engrafted with the aggressive murine AML cell line C1498 (Figure 4). Intriguingly, when combined with the autophagy inhibitor chloroquine, blueberry treatment substantially augmented the lifespan of these mice. The combination with chloroquine was selected in this instance after we previously observed and reported that C1498 cells up regulate autophagy as a mechanism of resistance to therapy [11]. Overall, these *in vivo* studies showed that dietary supplementation of blueberry extracts is sufficient to exert an anti-AML effect in animal models. This is important because pediatric and elderly AML patients may benefit from dietary therapeutic strategies that otherwise exert no toxicity.

Conclusion

There is an urgent need for more effective and less toxic therapies

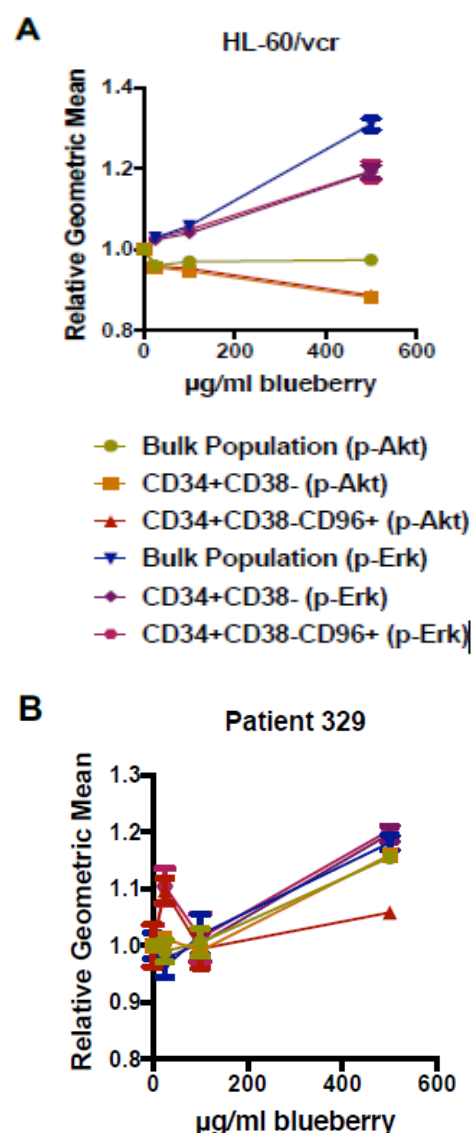


Figure 2: Blueberry extracts regulate Erk and Akt phosphorylation in leukemia stem cells.

(A) HL-60/vcr cells were exposed overnight (12 hours) to blueberry extracts and phosphorylation of Akt and Erk were monitored by Phosflow analysis (n=3 per data point). Antibodies were used to define leukemia stem cells as CD34 + CD38- or CD34 + CD38-CD96+.

(B) Phosflow analysis was similarly used to evaluate cells exposed overnight to blueberry extracts from AML patient case #329 (poor prognosis) (n=3 per data point).

for the treatment of AML. Many patients are not effectively treated with current standard of care therapies due to severe toxicity. Many times treatment will not commence, or will be halted before completion. Dietary interventions may offer alternative or adjuvants that are minimally toxic if at all. In the present study, blueberry extracts were evaluated for the first time for anti-AML efficacy in both cellular and animal models of AML. In addition to a demonstration of therapeutic efficacy in these models, the present study further demonstrated that blueberry extracts could regulate the AML stem cell. Therefore, constituent components of the blueberry hold promise not only as anti-AML therapeutics, but as anti-AML stem cell therapeutics. As part of an extract, or isolated and in combination with cytotoxic chemotherapeutics, blueberry components hold the potential to eradicate the specific cells that are primarily responsible for AML development, progression, relapse, and therapy resistance. More so,

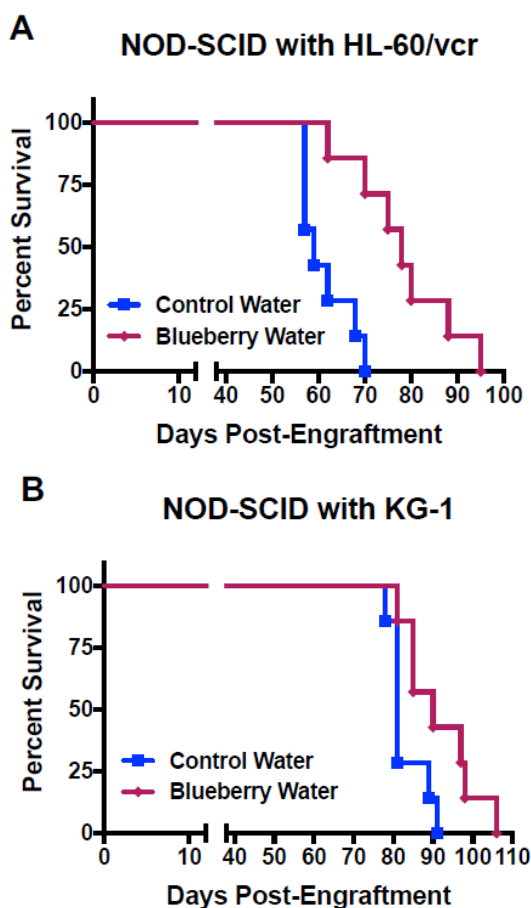


Figure 3: Survival extended in mice with AML following dietary blueberry extract supplementation.

A. NOD-*Scid* mice were xenografted with the human AML cell line HL-60/vcr (2×10^6 cells/mouse by tail vein injection) and survival was monitored after mice were given crude blueberry extract (1% w/v) (dark line with diamonds) *ad libitum* in the drinking water, or control water (light gray line with squares), starting one week following engraftment (Mantel-Cox Logrank test, $p=0.0017$, $n=7$ /group).

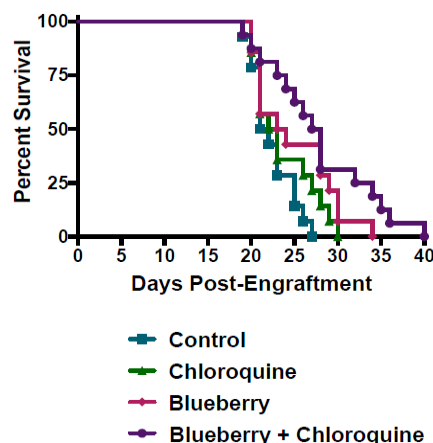
B. NOD-*Scid* mice were xenografted with the human AML cell line KG-1 (2.5×10^6 cells/mouse by tail vein injection) and survival was monitored after mice were given crude blueberry extract (1% w/v) (dark line with diamonds) *ad libitum* in the drinking water, or control water (light gray line with squares), starting one week following engraftment (Mantel-Cox Logrank test, $p=0.0382$, $n=7$ /group).

the success of the *in vivo* studies further showed good bioavailability for the components of the blueberry that exert anti-AML efficacy. Altogether suggesting that blueberries could be a profound source for anti-leukemia biopharmaceutical discovery and development.

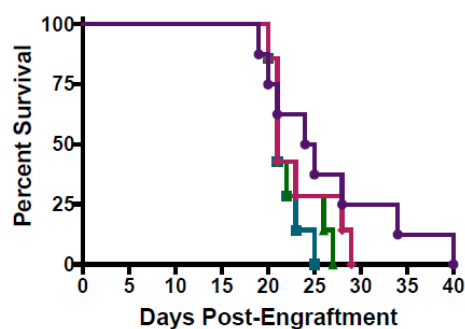
Finally, we have demonstrated that blueberry extracts can impede pro-inflammatory signaling pathways mediated by a neutral sphingomyelinase, as well as by an NADPH oxidase [3,4]. These particular pathways may augment leukemogenic signaling by redox-mediated inactivation of tyrosine phosphatases, which can lead to increased tyrosine kinase-mediated signaling [18,19]. In addition to the prevalence of dysregulated growth factor signaling

Pathways in AML and other myeloid hematological disorders, inflammatory cytokines have been associated with relapsing AML and AML secondary to Fanconi anemia [10,20-24]. Altogether, the persistence of these conditions in AML suggests that novel anti-inflammatory agents such as those derived from blueberry extracts may be of particular benefit for the treatment of AML. In conclusion,

Male and Female C57BL/6J with C1498



Male C57BL/6J with C1498



Female C57BL/6J with C1498

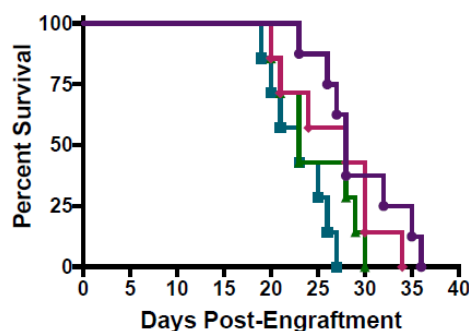


Figure 4: Dietary blueberry extract supplementation in combination with chloroquine extended the survival of mice with AML.

C57BL/6J mice were engrafted with the murine AML cell line C1498 (1×10^6 cells/mouse by retro-orbital injection) and survival was monitored after mice were given a crude blueberry preparation to eat (25 mg/mouse/week) (line with upside down triangles), chloroquine in the drinking water (25 μ M sweetened with 1% w/v glucose to alleviate bitterness) *ad libitum* (line with right side up triangles), or a combination of both (line with circles), or control water alone (line with squares), starting one week following engraftment (Mantel-Cox Logrank tests: Males & Females together, $p=0.0263$, $n \geq 14$ /group; Males only, not significant at $p=0.1225$, $n \geq 7$ /group; Females only, $p=0.0479$, $n \geq 7$ /group).

this study demonstrated anti-AML efficacy for blueberry extracts. This is of significance because natural products have long served as sources and blueprints for the discovery and development of novel biopharmaceuticals [25-27]. Therefore, the present study may hold promise to identify minimally toxic and potentially combinatorial anti-AML compounds that can be utilized in anti-AML biopharmaceutical development.

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